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Comparison of Use of Agglutination of Mammalian Erythrocytes Plus Heat Stability Test and Use of Agglutination of Mammalian Erythrocytes Plus Erythrocyte Elution Time to Characterize Isolates of *Newcastle Disease Virus*

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Authors' contributions

This work was carried out in collaboration between all authors. Author MCOE designed the experiments and also drafted the manuscript while authors JAOO, IEE, IJM and AAN conducted the laboratory experiments. All authors read and approved the final manuscript

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ABSTRACT

Aim: For a simpler, more rapid and more accurate method of characterizing new isolates of *Newcastle disease virus* (NDV), agglutination of mammalian erythrocytes (HA) plus heat stability test and agglutination of mammalian erythrocytes plus erythrocytes elution time (EET) were compared with use of Intra-cerebral pathogenicity index (ICPI) to characterize the isolates.
Materials and Methods: NDV isolates characterized by their ICPI were re-characterized by HA of mammalian erythrocytes plus heat stability test and by HA of mammalian erythrocytes plus EET. Rate of agreement of each of the two combinations with ICPI was calculated.
Results: HA of mammalian erythrocytes plus heat stability agreed with ICPI in

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characterizing 10 of the 12 NDV isolates (83.3%) while use of HA of mammalian erythrocytes plus EET agreed with ICPI in all the 12 isolates (100%).
Conclusion: It was concluded that use of combination of agglutination of mammalian erythrocytes and EET to characterize NDV isolates has better agreement with use of ICPI to characterize the virus than use of combination of agglutination of mammalian erythrocytes and heat stability test.

Keywords: Variation in erythrocyte elution time; Newcastle disease virus strains; characterization.

ABBREVIATIONS

ICIPI=Intracerebral Pathnogenicity Index; EET=Erythrocyte Elution Time; MRBCHA= Agglutination of mammalian RBC; HS=Heat stability; MRBCHA+HS=Combination of agglutination of mammalian RBC and heat stability test; MRBCHA+EET=Combination of agglutination of mammalian RBC and erythrocytes elution time; S=Heat stable; US=Unstable to heat; L=Lentogenic strains; M=Mesogenic strains; V=Velogenic strains.

1. INTRODUCTION

Newcastle disease (ND) is a contagious disease of domestic and wild avian species, characterized by gastrointestinal, respiratory and nervous signs. It is caused by Paramyxo virus sero-type 1 that is of the genus, Avulavirus, of sub family, Paramyxovirus, of family Paramyxoviridae and of the order, Mononegavirales [1-4]. The disease was first described in Newcastle Upon-Tyne, England [5]. In Nigeria, ND was first reported from Ibadan [6] and it has since then been reported in other parts of the country. It is enzootic in both local and commercial poultry [7-10].

Epidemiological studies and reports from some parts of Nigeria have rated Newcastle disease as one of the greatest constraints to development of both rural and commercial poultry [11] in the country. It has led to closure of many poultry farms. So, it is a cause of low output in poultry production and aggravates poverty [11]. The disease occurs all year round in Nigeria with the highest number of outbreaks occurring between November and January [8,13].

Pathogenicity of NDV isolates is a function of their haemagglutinin antigen (H-antigen) [14-16]. The H-antigen is also responsible for type of red blood cells (RBC) NDV strains agglutinate [17]. All NDV strains agglutinate chicken RBC [18-20] but in addition to chicken RBC the lentogenic strains agglutinate mammalian RBCs [17]. Also, the bonds formed between NDVH-antigen and RBCs in the process of haemagglutination clip off after some time by a process called erythrocyte elution.

Neuraminidase antigen is responsible for erythrocyte elution [21]. The H-antigen and neuraminidase antigen form a complex on envelopes of *Newcastle disease virus* and it has been observed [22] that NDV strains with high H-antigen activities (pathogenicity and heat stability) exhibit low neuraminidase activity (viral elution). Velogenic strains which are the most pathogenic strains also have H-antigens that are stable to heat (56℃ for 15 minutes) while the Mesogenic and Lentogenic strains are less pathogenic and their H-antigens lose activity when heated at 56℃ for 15 minutes [1,20] .

Determination of Mean death time (MDT) of chick embryos is the method often used in characterizing isolates of NDV strains in laboratories [23,24,12]. Velogenic NDV, kills chicken embryos in less than 60 hours. Mesogenic strains kill the embryos in 60-90 hours while Lentogenic strains take more than 90 hours to kill chicken embryos.

Untill recently there was no effective antiviral treatment for ND [25]. So, protection of poultry flocks against the disease has been mainly through vaccination and good bio-security measures [26-28]. Vaccination failures associated with introduction of new strains of the virus and wrong laboratory diagnosis of strains of NDV involved in outbreaks of the disease in different countries are often reported [29,30]. Need to characterize NDV isolates in each environment and in each outbreak therefore exists.

It had earlier been reported that differences exist between mean elution time of the three main strains of NDV [21]. These differences in EET can be used for quick characterization of NDV isolates in the field.

Laboratory methods used to characterize ND isolates also include intra-cerebral pathogenicity index, intravenous pathogenicity index (IVPI) and a combination of agglutination of mammalian erythrocytes and heat stability test [1,31].

To determine MDT, IVPI or ICPI involves inoculating new isolates into live chicks or into chicken embryos. The different NDV strains give different pathologic lesions in live chicks and in chick embryos. By their MDT and the lesions new NDV isolates are characterized [23,24,12]. These methods of viral characterization require skill, sterile conditions and laboratory setups that are often not available in the field. They also violate ethical regulations, in force in some countries.

Simpler and more rapid methods of characterizing NDV isolates that can be adopted in the field include , a combination of their ability to agglutinate mammalian RBCs and ability of their haemagglutinin antigens to withstand heat treatment and a combination of agglutination of mammalian RBCs and determination of their EET.

To decide which of these two simpler, less expensive , more rapid and ethical methods of characterizing NDV that can be adopted by less sophisticated laboratories, how much each of the methods agrees with use of ICPI to characterize NDV was assessed.

2. MATERIALS AND METHOD

Twelve NDV isolates, comprising, 4 of Velogenic strains, 4 of Mesogenic strains and 4 of Lentogenic strains characterized as such by the ICPI method (National Veterinary Resaerch Institute, Vom, Nigeria) were used for the study. Each of the isolates was tested for ability to agglutinate sheep RBCs and ability of its haemagglutinin to withstand 56℃ for 15 minutes [1]. Time (minutes) it takes each isolate to elute chicken RBCs after agglutination of the RBCs was also determined.

For the EET, HA test was done with 0.6% chicken RBC at room temperature to determine the time taken by RBCs in the control wells to settle while those in the test wells fail to settle (agglutination time). The setup was then incubated at 37℃ and observed till the RBCs eluted. Erythrocyte elution time of each sample was read as the interval between its agglutination time and the time it took RBCs in the well of its highest dilution which gave complete agglutination to elute.

The isolates were then re-characterized by combination of results of their ability to agglutinate mammalian erythrocytes plus result of their heat stability test and by combination of results of their mammalian RBC HA test plus their EET. Results of the two methods of characterization were matched with the strain they were earlier characterized into, by use of the ICPI method. Percentage agreement of each combination of methods with ICPI was calculated.

3. RESULT

Combination of agglutination of mammalian erythrocytes and heat stability test placed all the 4 Lentogenic isolates and all the 4 Velogenic isolates in the same strains ICPI placed them but of the 4 Mesogenic isolates, use of combination of agglutination of mammalian RBC and heat stability placed 2 as Mesogenic strain and the other 2 as Velogenic strains (83.3% agreement) while combining agglutination of mammalian erythrocytes with EET to characterize the viral isolates placed all the isolates in the same strains they were placed by use of ICPI, thus giving 100% agreement with ICPI Tables 1 and 2 .

Table 1. Erythrocyte Elution Time (minutes) of samples of the three major strains of *Newcastle Disease Virus* at 37℃

Samples	Lentogenic	Mesogenic	Velogenic
I	10	9	153
II	8	21	270
III	10	22	240
IV	9	17	290
Mean	9.25±0.96	17.25±5.91	238.25±60.43

EET of the Velogenic Newcastle disease virus strains was significantly (P<0.01) longer than those of the Lentogenic and the Mesogenic strains while there was no significant difference (P>0.05) between EET of the Lentogenic and the Mesogenic strains

Table 2. Rate of Agreement of agglutination of mammalian erythrocytes+Heat stability test and Agglutination of mammalian erythrocyte+Erythrocyte elution time with use of intracerebral pathogenicity Index in characterizing Newcastle Disease Virus

NDVstrains (by ICPI)	MRBCHA	HS	EET(Min)	MRBCHA+HS	MRBCHA+EET
L	+	US	10	L	L
L	+	US	8	L	L
L	+	US	10	L	L
L	+	US	9	L	L
M	–	US	9	M	M
M	–	S	21	V	M
M	–	S	22	V	M
M	–	US	17	M	M
V	–	S	153	V	V
V	–	S	270	V	V
V	–	S	240	V	V
V	–	S	290	V	V

HA of mammalian RBC+Heat stability test agreed with ICPI in 10 out of the 12 samples (83.3%) while HA of mammalian RBC+EET agreed with ICPI in all the 12 samples (100%).

4. DISCUSSION

Newcastle disease has been reported in the United States of America, Europe, Asia and Africa [5,6]. In Nigeria and in many other countries it has become endemic. Prophylactic vaccination against the disease is practiced in all but a few of the countries that produce poultry on commercial scales [24].

One of the most characteristic properties of the different strains of NDV include variation in their pathogenicity to chickens. Pathogenicity is a function of the haemagglutinin antigen. Haemagglutinin antigen is also responsible for agglutination of RBCs by NDV which is an important test used to detect presence of NDV or presence of its antibodies [32]. The different strains of NDV also vary in their ability to agglutinate RBCs of different animal species and in ability of their haemagglutinin antigens to remain stable when heated at 56℃ for 15 minutes. So, these properties are used to characterize NDV isolates.

Methods of characterizing NDV isolates also include Plaque formation in cell cultures [21]. Heat stability test is one of the methods often used to characterize NDV in developing countries because, it is easy and cheap.

It has been reported that only the Velogenic strain has H-antigen that is heat stable [1] but in this study two samples classified as Mesogenic strain by use of the ICPI method (NVRI, Vom, Nigeria) were HA heat stable. Nawathe [1] had earlier reported that some Nigerian Mesogenic NDV isolates were pathogenic. Both heat stability used by Nawathe [1] and pathogenicity which ICPI uses are functions of the haemagglutinin antigen. Therefore, that some Nigerian Mesogenic isolates were heat stable suggests that they may also produce clinical Newcastle disease in susceptible chickens thus supporting the observation made by Nawathe [1].

Heat stability could not differentiate Nigerian Mesogenic isolates from the Velogenic NDV strains in this study. So, combination of agglutination of mammalian RBC and heat stability to characterize NDV which is the practice in most laboratories in Nigeria may be leading to wrong conclusions. This may be responsible for our earlier observation that healthy free roaming chickens in the country were carriers of heat stable NDV isolates [20].

As alternative to heat stability test, EET of new NDV isolates should be used to characterize them. EET of the Velogenic isolates was significantly longer ($P<0.01$) than those of the Mesogenic and Lentogenic strains. The Velogenic NDV isolates had EET that was consistently one hour or longer while EET of the Lentogenic and Mesogenic strains were only few minutes.

The total agreement (100%) that existed between use of a combination of agglutination of mammalian RBCs and EET to characterize NDV and use ICPI to characterize the virus confirms that the new method is reliable and should be adopted.

Both agglutination of mammalian RBCs and determination of EET can be run concurrently, on same plate, by using chicken RBC in a row and mammalian RBC in another row. So, the new method of characterizing NDV would be rapid and cheap.

Since temperature variation affects viral erythrocyte elution time, EET and agglutination of mammalian RBC, to characterize NDV should be run at a controlled temperature (37℃) in order to make results reproduceable.

5. CONCLUSION

Any isolate of *Newcastle disease virus* that fails to agglutinate mammalian erythrocytes and has EET of one hour or longer is likely to be a velogenic strain of the virus. Any that fails to agglutinate mammalian RBCs but has EET of less than one hour may be a mesogenic strain while those that agglutinate mammalian erythrocytes and have short EET are lentogenic strains.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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